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Interactions of Single and Combined Human Immunodeficiency Virus Type 1 (HIV-1) DNA Vaccines

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DNA immunization permits evaluation of possible antagonistic or synergistic effects between the encoded components. The protein expression capacity *in vitro* was related to the immunogenicity *in vivo* of plasmids encoding the HIV-1 regulatory genes *tat rev,* and *nef.* Neither Tat nor Rev expression was influenced by co-expression *in vitro* of all three proteins, while Nef expression was slightly inhibited. With the combination of genes, the T-cellular responses of mice against Rev and Nef were inhibited compared with those when single gene immunization was used. No interference was detected for the Tat T-cell response. Thus, co-immunization with certain genes may result in inhibition of specific immune responses.

Key Words. DNA immunization; regulatory genes; HIV; Tat; Rev; Nef; gene interaction; protein interaction.

INTRODUCTION

DNA immunization has been shown to generate humoral and cellular immune responses to a number of pathogens in various animal models and primates, including humans (Calarota *et al.,* 1998; Donnelly *et al.,* 1997; MacGregor *et al.,* 1998; Robinson *et al.,* 1993; Ugen *et al.,* 1998; Wang *et al.,* 1998). Only a few studies have been published on the use of combined genes or multivalent DNA vaccines (Braun *et al.,* 1998; Cardoso *et al.,* 1998; Grifantini *et al.,* 1998; Hinkula *et al.,* 1997a; McClements *et al.,* 1996). Co-immunization of mice with genes from the measles virus (Cardoso *et al.,* 1998) resulted in an antibody subclass switch from an IgG2a-type response elicited by nucleoprotein alone to an IgG1 response when combined with hemagglutinin. This indicates that co-expression of different proteins can interfere with the type of responses induced.

Recent data indicate that immune reactivity to the early proteins Tat, Rev, and Nef of human immunodeficiency virus type 1 (HIV-1) or recombinants between simian and human immunodeficiency virus (SHIV) may contribute to protection from primary infection or progression of disease (Cafaro *et al.,* 1999; Calarota *et al.,* 1998; Osterhaus *et al.,* 1999). High-level transcription from the integrated provirus is regulated by the viral protein Tat and several cellular cofactors (Bieniasz *et al.,* 1998; Parada and Roeder, 1999; Wei *et al.,* 1998). The viral protein Rev exports the intron containing viral mRNA transcripts from the nucleus to the cytoplasm (Feinberg and Greene, 1992) with the aid of several cellular cofactors (Fritz *et al.,* 1995; Murphy and Wente, 1996). The viral protein Nef downregulates both CD4 receptors and molecules of the major histocompatibility complex (MHC) class I from the surface of infected cells (Aiken *et al.,* 1996; Cohen *et al.,* 1999; Harris, 1996; Schwartz *et al.,* 1996). Nef is also suspected to disrupt normal T-cell signaling (Khan *et al.,* 1998; Piguet and Trono, 1999).

By inducing immune responses to the regulatory proteins of HIV-1, it should be feasible to prevent the burst of newly synthesized virions from infected cells and thereby limit primary and chronic infection. We have previously studied the immunogenicity of these genes in a combination with structural genes in several mouse strains (Hinkula *et al.,* 1997a,b). Not until now, however, has it been feasible directly to analyze the interactions between the plasmids on the level of gene expression and to study the influence on immunogenicity in the combination of plasmids. Quantification of the *tat*, *rev,* or *nef* gene expression in the combination of plasmids was not done in the previous study (Hinkula *et al.,* 1997b).

In the present study, we have studied the genes in identical backbone vectors, encoding the neomycin/kanamycin resistance gene, the polyA signal from human papilloma virus type 16 (HPV 16) in a pUC-8-derived vector backbone. We compared gene interaction at the level of protein expression with the immunogenicity *in*

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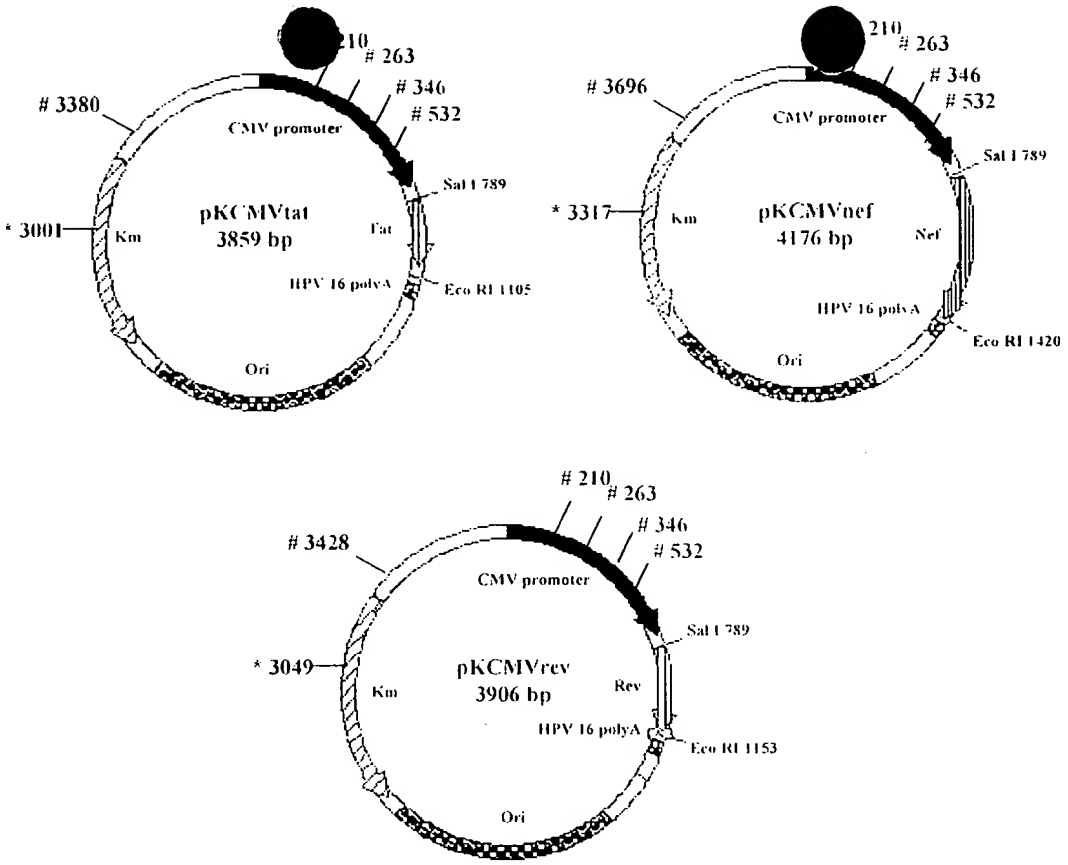

FIG. 1. Schematic drawing of the three DNA vectors used. All three plasmids have approximately the same length. Km, Kanamycin resistance gene; #, the nucleotide location of murine (GACGTC) immunostimulatory CpG motifs; *, the location of the human (AACGTCGA) CpG motif.

vivo to identify possible interference or synergy between the plasmids. Interference was detected in *nef* expression capacity when *tat*- and *rev*-encoding plasmids were present. The immunogenicity of the *rev* and *nef* genes was influenced by the presence of the other two proteins with decreased T cellular responses. We therefore suggest that administration of the early genes of HIV-1 as single immunogens should be considered for optimal protein expression and strong immunogenicity.

RESULTS

Cloning, plasmid characterization and in vitro expression

The coding sequences for full-length *tat*, *rev*, and *nef* of HIV-1 LAI were cloned into the vector pKCMV by PCR methods and were designated pKCMV*tat*, pKCMV*rev*, and pKCMV*nef* (Fig. 1). The plasmids were characterized by restriction enzyme analysis and sequence analysis of the promoter, insert, and polyA sequence. Single nucleotide changes were noted, though only one amino acid (aa) change had occurred; the Nef protein had a substitution at aa 133 from Ile to Thr. As Thr at aa 133 occurs in several viral isolates, the plasmid was accepted. Western blotting (Fig. 2) and indirect immunofluorescence (Fig. 3), using HeLa cells transfected with the different plasmid constructs and the empty vector as control, confirmed the *in vitro* expression of single plasmid formulations. Twenty-four hours after transfection, the majority of Tat, Rev, and Nef proteins could be detected in the cytoplasm. The molecular weight of Tat was found to be ~16 kDa, of Rev ~19 kDa, and of Nef ~27 kDa. The

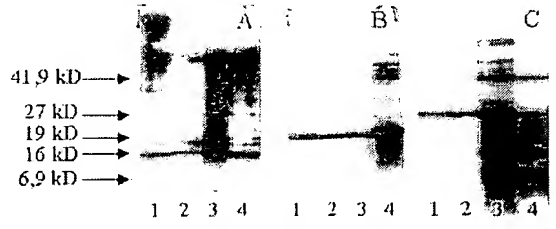

FIG. 2. (A) Tat expression from pKCMV*tat*. Expression was detected with Tat-specific mouse monoclonal antibodies in 1:500 dilution (1) or 1:1500 dilution (2) and with Tat specific rabbit polyclonal antibodies in 1:500 dilution (3) or 1:1500 dilution (4). (B) Rev expression from pKCMV*rev* detected with Rev-specific mouse monoclonal antibodies in 1:500 dilution (1) or 1:1500 dilution (2) and with Rev specific rabbit polyclonal antibodies in 1:1500 dilution (3) or 1:500 dilution (4). (C) Nef expression from pKCMV*nef* detected with Nef-specific mouse monoclonal antibodies in 1:500 dilution (1) or 1:1500 dilution (2) and with Nef-specific rabbit polyclonal antibodies in 1:1500 dilution (3) or 1:500 dilution (4). Detection was performed on HeLa cells transfected with the different constructs followed by detection using an ECL kit.

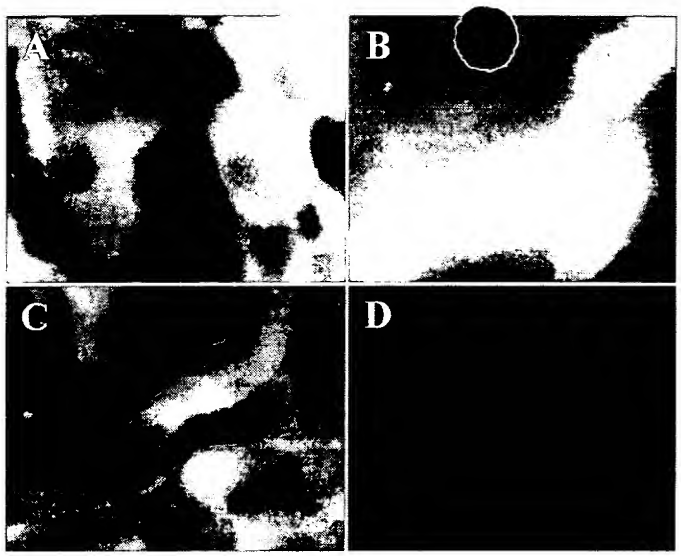

FIG. 3. Transient expression of Tat (A), Rev (B), Nef (C), and the empty vector pKCMV (D). Indirect immunofluorescence was performed on HeLa cells transfected with the pKCMV*tat*, pKCMV*rev*, pKCMV*nef*, or pKCMV constructs using polyclonal rabbit sera against the respective protein. The majority of protein expression was detected in the cytoplasm 24 h after transfection.

additional band seen close to the in the Tat Western blot is due to insufficient reduction of the internal disulfide bonds present in the Tat protein.

The presence of immunostimulatory motifs was investigated in pKCMV*tat*, pKCMV*rev*, pKCMV*nef*, and pKCMV. All plasmids contain five murine immunostimulatory motifs (GACGTC) and one human stimulatory motif (AACGTCGA) (Fig. 1). No immunostimulatory motifs were found in the *tat*, *rev*, or *nef* open reading frames.

Tat expression

Quantitative Tat-dependent CAT expression *in vitro* was examined in *tat* co-transfected HeLa cells, followed by detection of CAT protein by a CAT-specific ELISA (Fig. 4). No interference of Tat transcriptional activity was apparent when all three genes (*tat*, *rev*, and *nef*) were combined in the co-transfection assay compared to when the *tat* gene was expressed alone. HCMV*tat* (Calarota *et al.*, 1998) was included each time as a positive control. HCMV*tat* is identical to pKCMV*tat* except that the former encodes the ampicillin-resistance gene and the latter the kanamycin-resistance gene. Untransfected cells, pNL*CATw*, pBluescript, HCMV*tat* alone, pKCMV*tat* alone, and the mixture of the three pKCMV*tat*, pKCMV*rev*, and pKCMV*nef* coding vectors without pNL*CATw* were included as negative controls and did not result in CAT production.

Rev expression

Quantitative Rev-dependent p24 production *in vitro* was investigated in HeLa cells transfected with the *rev* gene followed by p24 detection (Fig. 5). The co-expression of Tat and Nef with Rev did not show any interference with the biological activity of the expressed Rev protein. The plasmid pB*rev* (the *rev* gene under the control of HIV-1 LTR, see Materials and Methods) was included as a positive control. Untransfected cells, pBluescript, HCMV*tat* + p24, pB*rev* + p24, pKCMV*rev* + p24, pKCMV*tat*, *rev*, and *nef* alone were included as negative controls. Positive p24 values in the control transfections

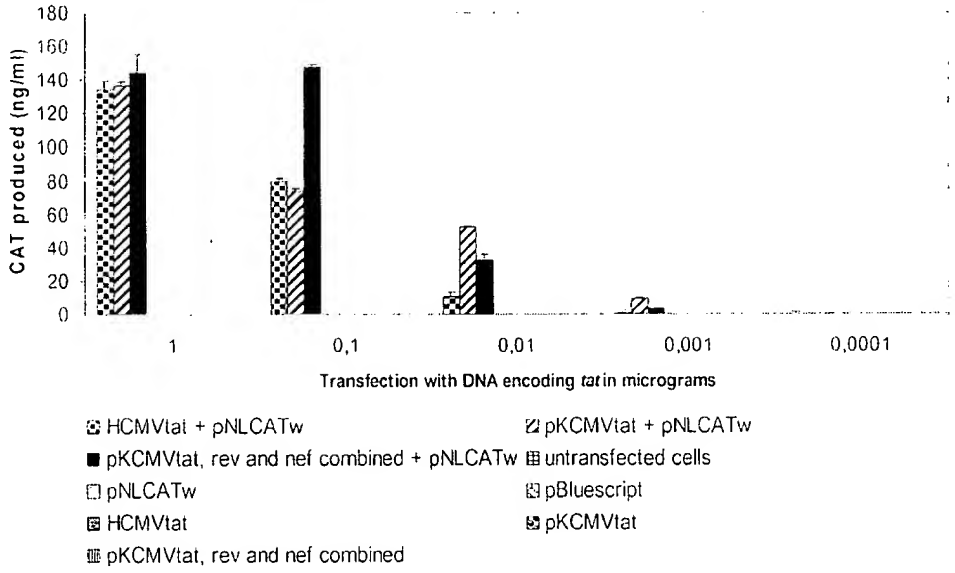

FIG. 4. Tat-dependent CAT expression *in vitro*. CAT expression was detected in HeLa cells co-transfected with the respective *tat*-encoding plasmid and CAT-expressing plasmid. *HCMVtat* was included as a positive control. Transfection with the pNL*CATw* plasmid was consistently performed with 0.5 μg of DNA. The pBluescript alone was transfected with 2 μg. The *tat*-encoding plasmids in the negative controls were transfected with 1 μg of DNA. In all cases, the total DNA amount was adjusted to 2 μg of DNA, using the pBluescript non-coding plasmid. The figure shows representative data of several experiments and standard errors. The single (▨) gene expression is compared with the combined (■) column.

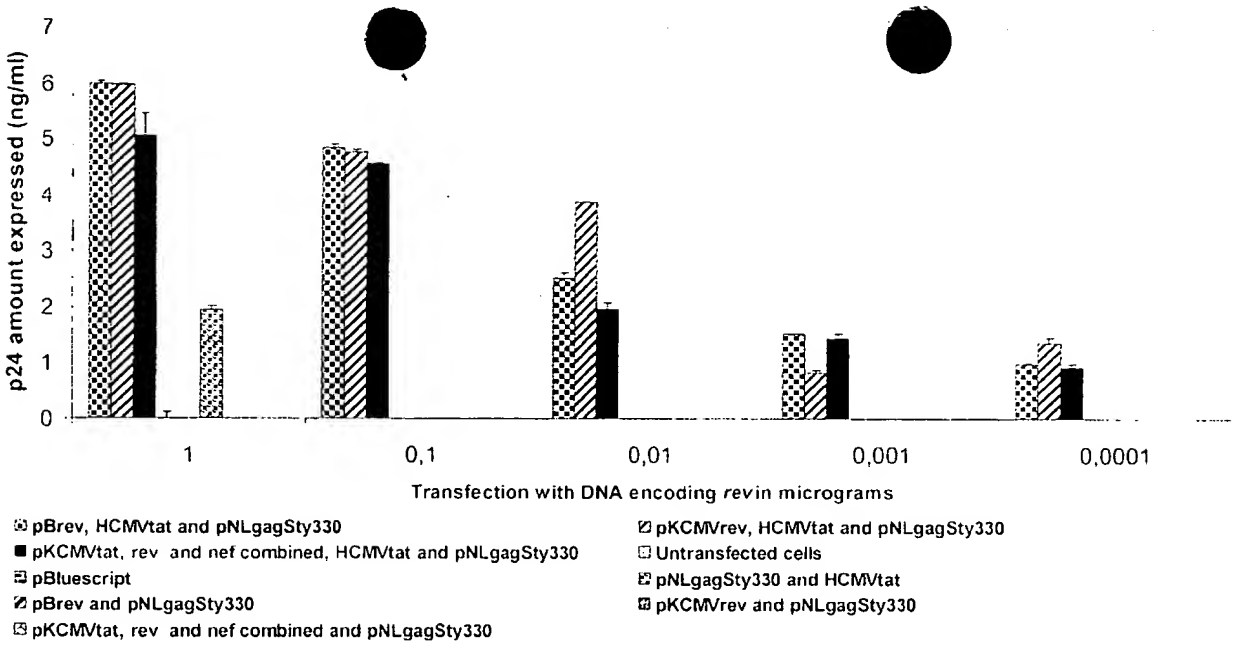

FIG. 5. Rev-dependent p24 expression *in vitro*. p24 is produced from cells co-transfected with the respective *rev*-encoding plasmid, a *tat*-encoding plasmid, and a p24-expressing plasmid. The plasmid pBrev is included as a positive control. Transfections with the p24- and *tat*-encoding plasmids were consistently performed with 0.5 μg of DNA. As negative controls, HeLa cells were transfected with either 2 μg of pBluescript DNA or 1 μg of the *rev*-encoding plasmid DNA alone. In all transfections, the total DNA amount was adjusted to 2 μg using the pBluescript noncoding plasmid. The figure shows representative data of several experiments and standard errors. The single (▨) gene expression is compared with the combined (■) column.

are due to the natural Rev-independent p24 production from the p24-encoding plasmid.

Nef expression

Nef *in vitro* expression capacity was evaluated in HeLa cells transfected with the *nef* gene, and a sandwich ELISA was used to detect Nef (Fig. 6). Protein expression was more efficient when the *nef*-encoding plasmid was used alone than when was it was combined with *rev*- and *tat*-encoding plasmids. HCMV*nef* was included as a positive control. Untransfected cells, empty vector pKCMV, and pBluescript were included as negative controls.

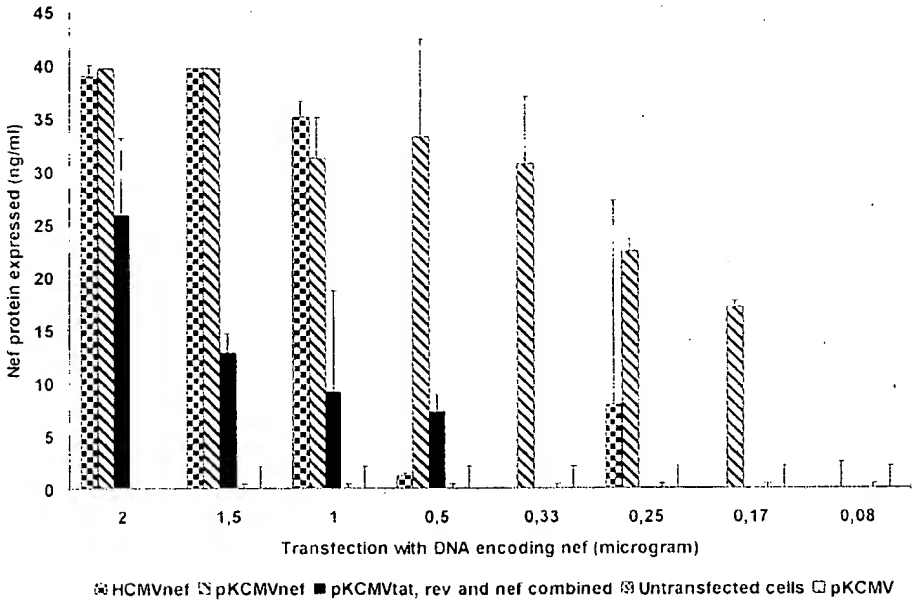

FIG. 6. Nef expression *in vitro*. Nef is produced from HeLa cells transfected with the respective *nef*-encoding plasmid. HCMV*nef* is included as a positive control. HeLa cells were transfected with either 2 μg of the empty vector pKCMV or 2 μg of pBluescript DNA as negative controls. In all transfections, the total DNA amount was adjusted to 2 μg using the pBluescript noncoding plasmid. The figure shows representative data of several experiments and standard errors. The single (▨) gene expression is compared with the combined (■) column.

TABLE 1

B-Cell Stimulation *in Vitro* to Tat, Rev, and Nef Antigens

Plasmid immunogen	No. of mice	DNA dose (μg)	Frequency (%) of Responders						
			rTat	Tat peptides	rRev	Rev peptides	rNef	Nef peptides	CAG
pKCMV*tat*	8	20–100	8/8 (100)	7/8 (88)	3/8 (38)	ND	ND	ND	0/8 (0)
pKCMV*rev*	8	20–100	4/8 (50)	ND	8/8 (100)	5/8 (63)	ND	ND	0/8 (0)
pKCMV*nef*	7	20–100	ND	ND	0/7 (0)	ND	3/7 (43)	7/7 (100)	ND[a]
pKCMV*tat*, *rev* and *nef* combined	18	3 × (20–100)	18/18 (100)	18/18 (100)	17/18 (94)	14/18 (78)	16/18 (89)	18/18 (100)	ND*

Note: Groups with >90% reactivity are shown in bold. Percentages are in parentheses.

[a] ND*, not done due to lack of cells.

Immunogenicity of mice immunized with tat,- rev,- or nef-expressing DNA

The biological effects of immunization were investigated in separate experiments in mice with single-gene administration and with the combination of pKCMV*tat*, *rev*, and *nef*.

Tat immunity

Humoral responses to Tat. Tat immunization by single genes elicited 100% responders to the rTat protein in mice (Table 1) by *in vitro* B-cell stimulation. The response to Tat representative peptides was measured, and 88% of the *tat* immunized mice responded. In the groups immunized with the combination of all three genes, all mice responded to both rTat and Tat peptides. There was thus no difference in the frequency or magnitude of the response between *tat* as a single gene and in the combination of *rev* and *nef* genes.

Epitope mapping of Tat peptide responses indicated that B-cell epitopes are strong at aa 31–65 (Fig. 7A). Tat immunization induced antibodies *in vitro* to the same major epitopes when administered as a single gene and in the combination formula.

DNA immunization did not result in significant IgG seroconversion against HIV-1 Tat except for very low titers in a few animals in each group. DNA immunization thus resulted in priming of relevant B cell clones, but the procedure was not sufficient to induce a complete antibody response. Subclass determination was performed on sera reacting positively in IgG ELISA. IgG1/IgG2a ratios of IgG responses to rTat and Tat peptides resulted in ratios between 1.9 and 2.9, indicating stimulation of both Th1 and Th2 responses (data not shown).

T-cell responses to Tat. The combination of all three genes elicited a higher frequency of responders to rTat and Tat peptides than did the pKCMV*tat* alone (Table 2). Only 25% of the mice immunized with the single *tat* gene responded to rTat and 63% to Tat representative peptides. Of the mice receiving the combination of genes, 78% responded to rTat and 94% to Tat representative peptides. As with the B-cell stimulation assay, the magnitude of response did not differ significantly between

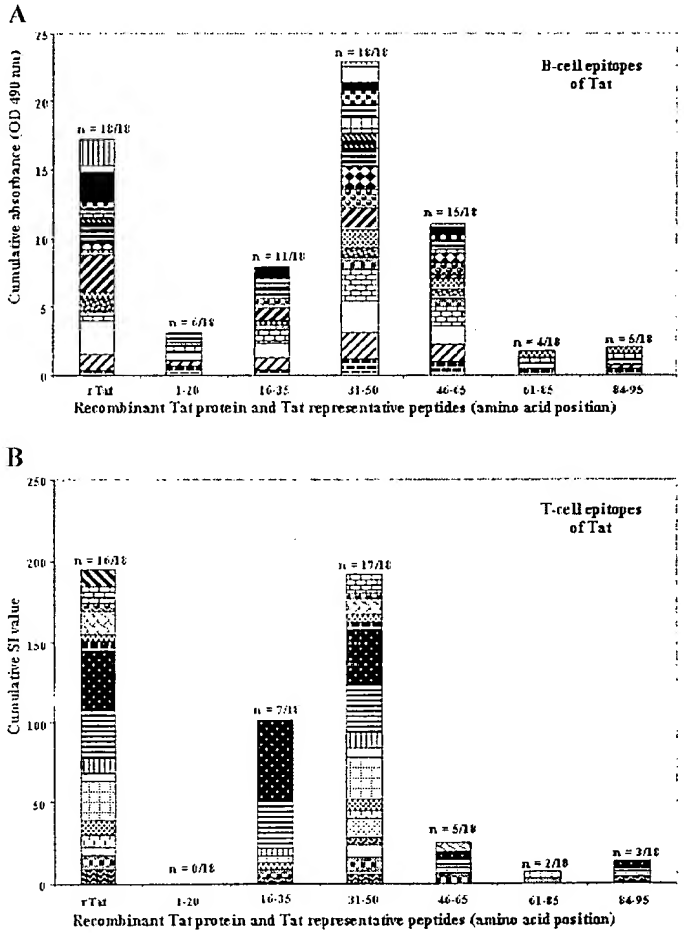

FIG. 7. B-cell and T-cell epitopes following *tat* immunization. (A) Frequency of B-cell epitope reactivity and mapping *in vitro* to recombinant Tat and Tat representative peptides in (C57BL/6)H2-b mice immunized with pKCMV*tat*, *rev*, and *nef* combined. The number of mice (*n*) reacting to the respective antigen is indicated. (B) Frequency of T-cell epitope reactivity and mapping *in vitro* to recombinant Tat and Tat representative peptides in (C57BL/6)H-2b mice immunized with pKCMV*tat*, *rev*, and *nef* combined. The number of mice (*n*) reacting to the respective antigen is indicated. Mean cpm for medium was 4946.

TABLE 2

T-Cell Stimulation *in Vitro* to Respective Antigen

Plasmid immunogen	DNA dose (μg)	No. of mice	Frequency (%) of Responders						
			rTat	Tat peptides	rRev	Rev peptides	rNef	Nef peptides	CAG
PKCMV*tat*	20–100	8	2/8 (25)	5/8 (63)	1/8 (13)	ND	ND	ND	1/8 (13)
PKCMV*rev*	20–100	8	0/6	ND	2/8 (25)	6/8 (75)	ND	ND	0/8 (0)
PKCMV*nef*	20–100	7	ND	ND	0/7 (0)	ND	7/7 (100)	5/7 (71)	2/7 (29)
pKCMV*tat*, *rev* and *nef* combined	3 × (20–100)	18	14/18 (78)	17/18 (94)	5/18 (28)	8/18 (44)	13/18 (72)	11/18 (61)	0/18 (0)
PKCMV	300	5	2/5 (40)	1/5 (20)	0/5 (0)	1/5 (20)	0/5 (0)	0/5 (0)	0/5 (0)

Note: Groups with >90% reactivity are shown in bold. ND, not done. Percentages are in parentheses.

[a] Mice immunized with 50 μg followed by 10 μg pKCMV*rev* not rTat analyzed.

mice immunized with single gene and mice immunized with the combination of genes.

Epitope mapping analysis revealed strong T-cell epitopes between aa 16 and 50 (Fig. 7B). The epitopes were shared by mice immunized with single *tat*-encoding plasmid and mice immunized with the combination of all three plasmids. However, stronger reactions to the epitopes were observed in mice that had received the combination of plasmids.

Rev immunity

Humoral responses to Rev. Single *rev* gene administration of 20–100 μg to mice elicited 100% responses in *in vitro* B-cell stimulation to the rRev protein (Table 1) and 63% responders to Rev peptides.

The response to rRev elicited with the combination of genes was as efficient as single *rev* gene administration. However, the number of B-cell stimulation responders to Rev peptides was slightly higher in the mice that had received the combination of all three genes. The magnitude of the response to rRev elicited by single *rev* gene immunization was, however, statistically stronger ($P = 0.002$, Mann–Whitney *U*-test) than that induced by the combination of genes (Fig. 8A).

Epitope mapping of Rev peptide responses indicated that B-cell epitopes are strong at aa 18–50 (Fig. 8B). Rev immunization induced antibodies *in vitro* to the same major epitopes when administered as a single gene and in the combination formula.

DNA immunization did not result in significant seroconversion against HIV-1 Rev except for very low titers in a few animals in each group. DNA immunization thus resulted in priming of relevant B-cell clones, but the procedure was not sufficient to induce a complete antibody response. Subclass determination was performed on sera reacting positively in IgG ELISA. IgG1/IgG2a ratios of IgG responses to rRev and Rev peptides were between 2.2 and 3.9, indicating stimulation of both Th1 and Th2 responses (data not shown).

T-cell responses to Rev. Twenty-five percent of the mice immunized with the single *rev* gene showed specific T-cell proliferative responses to rRev and 75% to Rev-representative peptides. The DNA administration in the combination experiments elicited 28% responders to rRev and 44% responders to Rev representative peptides. The magnitude of the T-cell response to Rev peptides was stronger in mice that had received the single *rev* gene than in those that had received the combination of genes (Fig. 8C, $P = 0.002$, Mann–Whitney *U*-test).

The T-cell epitope pattern differed slightly between mice immunized with the single *rev* gene and those immunized with the combination. Single-gene immunized mice responded to epitopes present at aa 1–20 and 31–100 (data not shown), while mice immunized with the combination of all three genes responded to aa 18–50 (Fig. 8D).

Nef immunity

Humoral responses to Nef. Nef immunization using single *nef* gene administration to mice elicited 43% B-cell stimulation responders to rNef and 100% responders to Nef representative peptides (Table 1). Of the mice that received the combination of genes, 89% responded to rNef and 100% to Nef representative peptides. No difference in magnitude was seen between the single-*nef*-gene immunized mice and the mice receiving the combination of genes.

Epitope mapping of Nef peptide responses demonstrated that major epitopes are found at the C-terminal domain of the protein at aa 151–185 (Fig. 9A). Nef immunization induced antibodies to the same major epitopes when administered as a single gene compared with the combination except that the N-terminal epitope reactivity was more prominent in mice immunized with the single *nef* gene.

DNA immunization did not result in significant seroconversion against HIV-1 Nef except for very low titers in a few animals in each group. DNA immunization thus

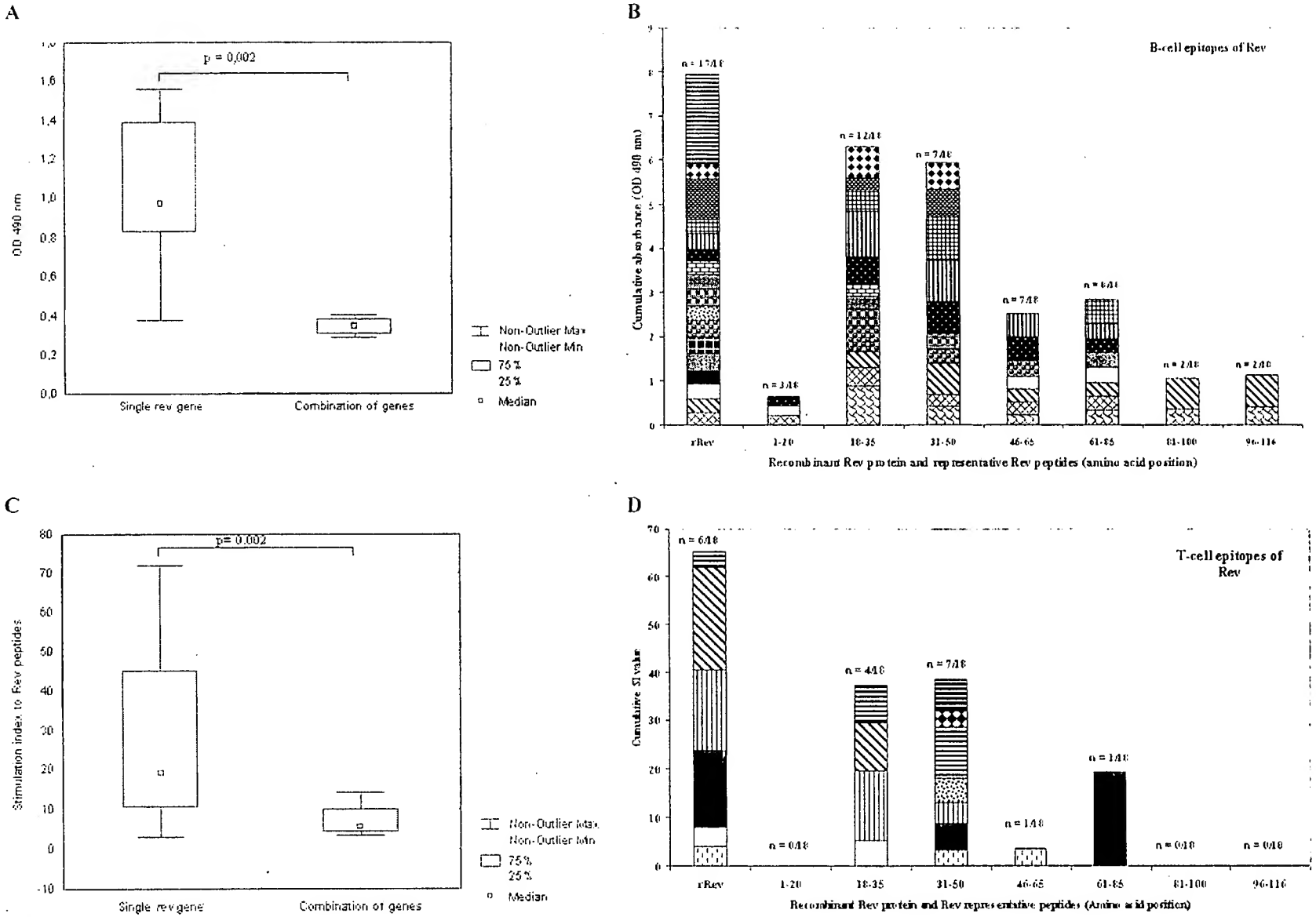

FIG. 8. B- and T-cell reactivity following *rev* immunization. (A) Magnitude of B-cell stimulation *in vitro* to rRev in mice immunized with the single *rev* gene or the combination of all three genes. Optical densities at 490 nm are shown. Reactions are considered positive above the cut-off value (geometric mean value of the naive control group plus 2 standard deviations). (B) Frequency of B-cell epitope reactivity and mapping *in vitro* to recombinant Rev and Rev representative peptides in (C57BL/6)H-2b mice immunized with pKCMV*tat, rev,* and *nef* combined. The number of mice (*n*) reacting to the respective antigen is indicated. (C) Magnitude of T-cell proliferative stimulation indices (SI) to Rev-specific peptides induced in mice immunized with the single *rev* gene or the combination of all three genes. All SI values 3 were considered positive. (D) Frequency of T-cell epitope reactivity and mapping *in vitro* to recombinant Rev and Rev representative peptides in (C57BL/6)H-2b mice immunized with pKCMV*tat, rev,* and *nef* combined. The number of mice (*n*) reacting to the respective antigen is indicated. Mean cpm for medium was 4946.

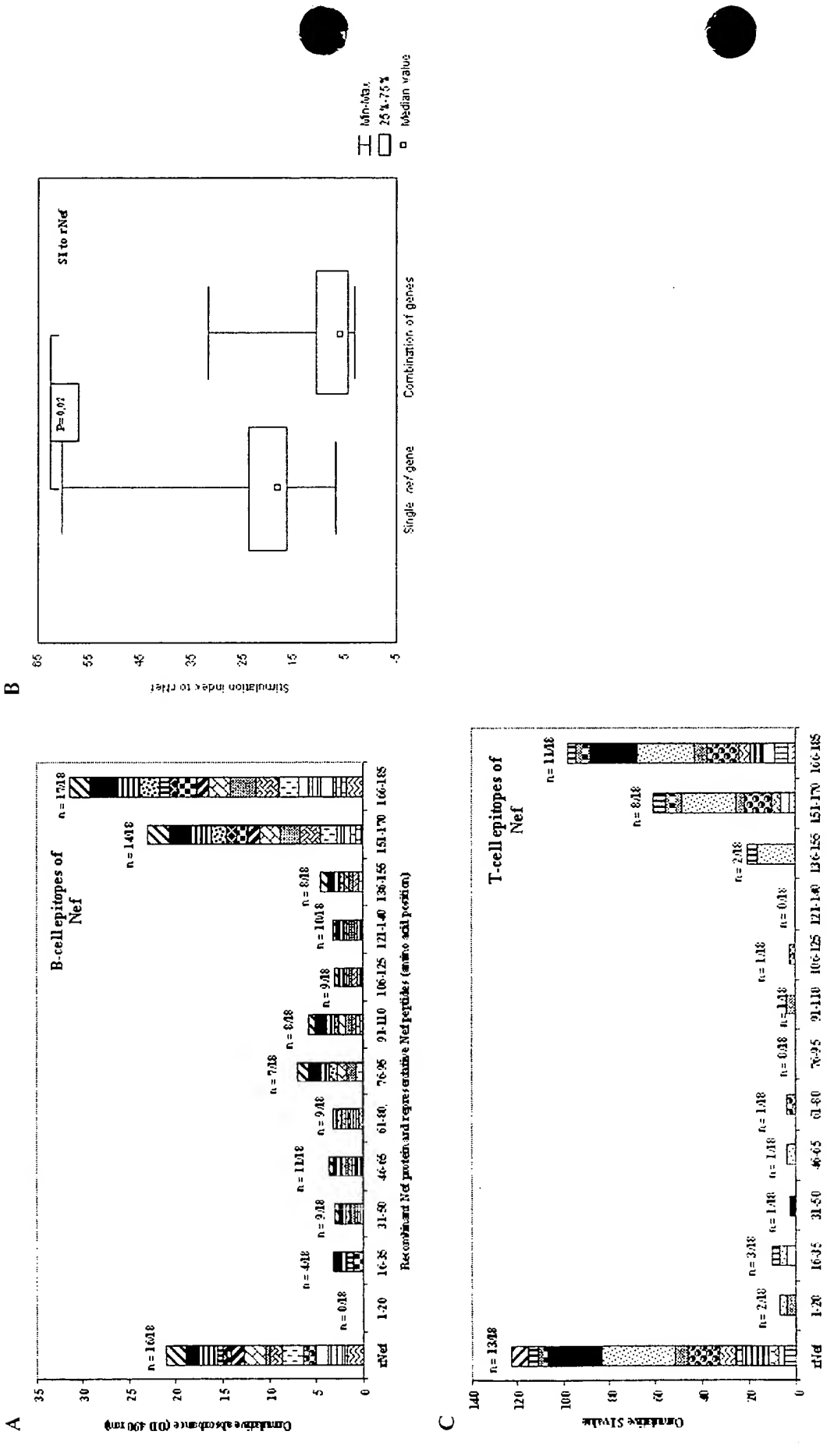

FIG. 9. B- and T-cell epitopes following *nef* immunization. (A) Frequency of B-cell epitope reactivity and mapping *in vitro* to recombinant Nef and Nef representative peptides in (C57BL/6)H-2b mice immunized with pKCMV*tat, rev,* and *nef* combined. The number of mice (*n*) reacting to the respective antigen is indicated. (B) Magnitude of T-cell proliferative stimulation indices (SI) induced in mice immunized with the single *nef* gene or the combination of all three genes. All SI values 3 were considered positive. (C) Frequency of T-cell epitope reactivity and mapping *in vitro* to recombinant Nef and Nef representative peptides in (C57BL/6)H-2b mice immunized with pKCMV*tat, rev,* and *nef* combined. The number of mice (*n*) reacting to the respective antigen is indicated. Mean cpm for medium was 4946.

TABLE 3

Protein Expression Capacity Compared with Frequency of Responders to Tat, Rev and Nef Peptides or Recombinant Protein Antigens

Antigen	Immunogen	Protein expression capacity (%)[a]	Frequency of B-cell stimulation responders (%)	Frequency of IgG antibody responders (%)	Th1/2 ratio	B-cell epitope pattern, aa	Frequency of T-cell stimulation responders (%)	T-cell epitope pattern, aa
Tat	pKCMV*tat*	56 ± 28	100	88*	>1	31–50	88	31–50
	All genes combined	85 ± 43	100	100*	>1	31–65	100	16–50
Rev	pKCMV*rev*	93 ± 19	100	63*	>1	46–65	75	31–85
	All genes combined	109 ± 60	100	100*	>1	18–50	50	18–50
Nef	pKCMV*nef*	222 ± 123	100	100*	>1	1–20, 151–185	100	151–185
	All genes combined	76 ± 22	100	100*	>1	151–185	78	136–185

Note: Magnitude of antigen specific responses is not considered.

*Responses to both protein and peptides are summarized.

[a] Values related to standardized expression at several doses of plasmid.

resulted in priming of relevant B cell clones, but the procedure was not sufficient to induce a complete antibody response. Subclass determination was performed on sera reacting positively in IgG ELISA. IgG1/IgG2a ratios of IgG responses to rNef and Nef peptides resulted in ratios between 1.3 and 2.6, indicating stimulation of both Th1 and Th2 responses (data not shown).

T-cell responses to Nef. All mice immunized with single pKCMV*nef* (20–100 μg) responded to rNef (Table 2) and 71% to Nef representative peptides in the T-cell stimulation assay. Immunization with the combination of plasmids elicited less responders to rNef (72%) and to Nef representative peptides (61%). Single *nef* gene administration thus resulted in a slightly higher number of responders to rNef but also induced higher specific stimulation to rNef than when *nef* was combined with the other two genes ($P = 0.02$, Mann–Whitney *U*-test; Fig. 9B).

Nef epitope mapping of T-cell stimulation responses showed that the strongest cellular epitopes were present at aa 151–205 (Fig. 9C).

All immune responses to Tat, Rev, and Nef were compared to protein expression levels (Table 3) and a concordance between interference at the level of protein expression and interference of immunity was identified.

Unspecific responses

The empty vector pKCMV by itself elicited B- and T-cell activation measured as responses to the Tat and Rev antigens but not to the Nef antigens (Tables 1 and 2). Forty percent of the mice responded to rTat protein and 20% to Tat peptides. No mice responded to rRev, but 20% of mice showed Rev peptide-specific responses. None of the mice responded to control antigen, however. The plasmid preparation did not contain any lipopolysaccharides. All these putatively unspecific responses have been deducted from the above interpretations, but it is conceivable that they represent an activation by these plasmids of cross-reactive antibodies/T cells in the mice.

DISCUSSION

To arrive at an optimal combination of genes, more needs to be known about the interference between the different genes in a multivalent vaccine both *in vitro* and *in vivo*. In this study, we investigated the interference between the HIV-1 regulatory genes with respect to *in vitro* protein expression capacity and *in vivo* immunogenicity.

Previously, we have performed two studies where we characterized the immune responses to the regulatory proteins of HIV-1 (Hinkula *et al.*, 1997a,b). We evaluated epitope pattern, the kinetics of immune responses, and efficacy of different DNA dose responses using a combination of the regulatory proteins and three structural proteins of HIV-1 envelope (gp160) and gag (p24+p17). Further, different vector backbones and polyA signals were used in these two studies. All vectors encoded the human cytomegalovirus (CMV) immediate early promoter and the ampicillin resistance gene, but different polyA signals were used. No studies to evaluate possible antagonism or synergy between components in the combination were performed. Structural HIV-1 genes were also included in the mixture to mirror the HIV genome, possibly influencing responses to the regulatory genes. Different mouse strains were also used to evaluate efficacy of induction of humoral and cellular responses.

Our present data show that Tat and Rev production and biological activities were not affected by the co-expression of the other two HIV-1 regulatory proteins (Table 3). This seems consistent with the situation *in vivo*, where Tat and Rev exert their functions in the presence of each other and in the presence of Nef.

However, co-expression of Nef *in vitro* with the Tat and Rev proteins, did result in a slight decrease of Nef expression in our titration studies (Table 3). One may hypothesize that competition for transcription factors present in the transfected cells, or differences in the preferred microenvironment around the promoter, will

lead to decreased Nef expression lev[redacted]Since all three genes in this study used the same pr[redacted]oter and polyA signal, enhancer elements unknown to us might be present that benefit Tat and Rev expression. In HIV infection, all three proteins are expressed from the same set of multiply spliced mRNA early in infection. Nef expression is found in 80% of all transcripts, and one would have assumed that there would be no interference with Nef expression since it lacks HIV mRNA transcripts with introns and since the Tat and Rev proteins are present.

In vivo studies were also performed regarding the antigenicity of the genes as single or combined formulations. Immune responses to the Tat protein were slightly enhanced by the presence of the *rev*- and the *nef*-encoding plasmids. One explanation would be the increased concentration of murine CpG motifs present when the plasmids were mixed. The amount of the specific genes was kept constant during these experiments but not the total amount of DNA. Immunization with unmethylated CpG motifs has been shown to stimulate natural killer cells, macrophages, B cells, and dendritic cells *in vivo* and *in vitro* (Behboudi *et al.*, 2000; Hartmann and Krieg, 2000; Klinman *et al.*, 1999; Sato *et al.*, 1996; Weiner *et al.*, 1997). All three plasmids and the empty plasmid contain five murine immunostimulatory motifs (GACGTC) and one human stimulatory motif (AACGTCGA). Thus, the empty vector functions as the control for the innate, unspecific stimulation activated by the DNA plasmids *in vivo*.

Subclass determination indicated that both a Th1 type and a Th2 type of response were stimulated as expected using DNA vaccination. In our previous paper (Hinkula *et al.*, 1997b), we reported that at least one booster immunization was required to obtain a detectable humoral IgG responses to the Tat, Rev, and Nef proteins. However, in this paper we saw that memory B cells were induced already by a single injection of the relevant gene. T-cellular responses could be detected already after the primary DNA immunization with the regulatory genes. We also found strong Tat B- and T-cell epitopes between aa 31 and 65; this is consistent with previous findings (Hinkula *et al.*, 1997b). Since Tat is found both extracellularly and intracellularly, both humoral and cellular responses specific for Tat are desirable.

The elicitation of immune responses to the Rev protein was affected by co-expression of the Tat and Nef proteins. Both B-cell stimulation reactivity and T-cell stimulation indices were slightly stronger with the single *rev* gene compared with the combination formula. The epitope pattern to Rev indicated strong B- and T-cell epitopes between aa 18 and 50, similar with single and combined genes. Since Rev is found mainly intracellularly, a strong cellular response toward Rev is desired.

When the *nef* gene was combined with the *tat* and *rev* genes, Nef T-cell responses were reduced. An individual decrease in Nef expression was observed in each set of experiments for pr[redacted] expression capacity. T-cell responses were decr[redacted]ed by ~30% by rNef and by 40% by Nef peptides. We believe that we see an effect of combining the *tat*, *rev*, and *nef* genes of HIV-1 as separate plasmids. To obtain the best immune response to Nef, one should thus consider administering the *nef* and *rev* genes alone or in combination with structural HIV genes.

Epitope patterns to Nef were similar in mice immunized with single and combined plasmids, showing aa reactivity with the strongest epitopes at aa 151–185. Since Nef molecules are found intracellularly and secreted from the cell, both cellular and humoral immunities are desired against Nef.

A study from Ayyavoo *et al.* (2000) describes the use of a novel DNA vaccine cassette expressing three HIV-1 accessory genes under the control of a single promoter. These authors investigated combined DNA vaccination using a single plasmid encoding the three genes *vif*, *vpu*, and *nef* of HIV. In their paper, no interference was observed in the response obtained after the combined plasmid immunization compared to when using the single genes. Using a cassette encoding and expressing the three regulatory genes of HIV-1 seems applicable to avoid the interference we observed when using three different plasmids. It would be more cost-effective to develop cassettes compared to plasmids encoding single genes.

The regulatory proteins of HIV have been shown to influence a variety of host cell proteins that might interfere with antigen presentation and capability of the host to mount immune responses. Especially the Tat and Nef proteins influence cells important for the immune system. Nef downregulates both the CD4 receptor and MHC class I molecules from the surface of infected cells (Aiken *et al.*, 1996; Cohen *et al.*, 1999; Harris, 1996; Schwartz *et al.*, 1996). Expression of Nef might lead to reduced viral epitope presentation by MHC class I molecules important for CTL recognition, and Nef would also reduce interactions between CD4 and MHC class II molecules between different effector–target cells. Nef expression thus leads to reduced immune cell activation. Tat induces Fas expression, suggesting a role of Tat in Fas-mediated B-cell death (Huang *et al.*, 1997). Both Tat and Nef have been shown to influence the interleukin–2 (IL-2) receptor surface expression; IL-2 is the major cytokine for proliferation and differentiation of T lymphocytes. Since no upregulation of the IL-2 receptor is found in HIV infected patients, Tat and Nef are suspected to disturb the IL-2 and IL-2 receptor communication (Greenway *et al.*, 1994; Puri *et al.*, 1995). All these effects of the early proteins make them attractive targets for immunization.

To our knowledge, this is the first report to show that co-immunization with three genes that are co-expressed in the natural virus infection can result in inhibition of

both the protein expression efficacy [redacted] the development of a specific immune response to one of the components. This finding indicates that it is possible to create potent multivalent vaccines but that the components need to be compatible at the level of protein expression *in vitro* and immunogenicity *in vivo*.

MATERIALS AND METHODS

Plasmid constructs

The *nef* gene originates from the HIV-1strain HXB3 (Accession No. M14100 and X03188), while the *tat* (Accession No. K03455 and M19921) and *rev* (Mermer *et al.*, 1990) genes originate from the HXB2 strain of HIV-1. The genes were amplified by PCR (Perkin–Elmer/Cetus) from HCMV*tat*, HCMV*rev*, and HCMV*nef* (Calarota *et al.*, 1998, 1999), using synthetic oligonucleotide primers (Life Technologies Inc., Rockville, MD), which corresponded to 5′and 3′ end-flanking sequences for the different genes and contained *SalI*/*Eco*RI restriction sites. The fragments were inserted into the pKCMV vector, creating the plasmids pKCMV*tat*, pKCMV*rev*, and pKCMV*nef*. The pKCMV vector contains backbone sequences derived from pUC8, and the antibiotic selection marker is neomycin/kanamycin dependent (kanamycin kinase, EC No. EC2.7.1.95). The promoter used is the immediate early promoter from human CMV and the polyA signal originates from HPV 16 (Fig. 1). Plasmids were grown in *Escherichia coli* (*E. coli*) DH5α (Life Technologies Inc.) and was purified by anion exchange resin (Qiagen GmbH, Hilden, Germany). Concentration and purity were assessed spectrophotometrically, and constructs were confirmed by restriction enzyme digestion followed by TBE agarose gel electrophoresis. Promoter, gene, and polyA sequences were analyzed with an ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction kit on an ABI Prism 310 genetic analyzer (PE Biosystems, Foster City, CA).

Western blot analysis

Protein expression from the plasmids encoding the single genes was confirmed by Western blot (Fig. 2) using an ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). At 18–20 h before transfection, 200,000 HeLa cells were plated with complete Eagle MEM 1× (2 mM L-glutamine, 0.8 g/L sodium bicarbonate, 50 μg/L penicillin/streptomycin) and 10% fetal calf serum (FCS) (Life Technologies Inc.) in each well of a six-well plate. The cells were incubated at 37°C in 5% CO_2 for 24 h. Transfection was performed with FuGENE 6 transfection reagent (Roche Diagnostics Corp., Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Two micrograms of the corresponding DNA was used for the assay. The transfected cells were re-incubated for 24 h. The cells were washed twice with ice-cold PBS [redacted] being harvested in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and the proteins separated on a 12–15% SDS-ready gel (Bio-Rad Laboratories). The proteins were blotted to nitrocellulose using a transfer buffer (25 mM Tris/192 mM glycine in 20% methanol) at 100 V for 2–3 h. The blots were then blocked with 10% dried milk in PBS for 1 h at RT before being washed three times in 0.5% bovine serum albumin (BSA) with 0.5% Tween 20 in PBS. The strips were then hybridized to rabbit anti-Nef, anti-Tat, or anti-Rev polyclonal serum or mouse anti-Nef, anti-Tat, or anti-Rev monoclonal antibodies (mab) (Ovod *et al.*, 1992; Ranki *et al.*, 1994) overnight at RT. The nitrocellulose strips were washed and incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulins or goat anti-rabbit immunoglobulins (DAKO A/S, Glostrup, Denmark), both diluted 1/10,000, for 2 h at RT. After a final wash, the membranes were soaked in ECL solution (Amersham Pharmacia Biotech Inc.) and developed on film.

Immunofluorescence

Forty-eight hours before transfection, one chamber slide with cover (Lab–Tek, Nalge Nunc International, Naperville, IL) was washed in 20% HCl at + 4°C overnight. The plates were washed with sterile water six times before being seeded with 0.5 ml/well of 2×10^6 HeLa [in Eagle's MEM 1× with 2 mM L-glutamine, 0.8 g/L sodium bicarbonate, 50 μg/L penicillin/streptomycin, and 10% fetal calf serum (FCS), Life Technologies Inc.]. The cells were incubated at 37°C in 5% CO_2 for 24 h. Transfection was performed with FuGENE 6 (Roche Diagnostics Corp.) according to the manufacturer's instructions. Two micrograms of the corresponding DNA was used in this assay. The transfected cells were re-incubated for 24–48 h. The cells were washed twice with ice-cold PBS before being fixed with 2% paraformaldehyde in PBS at +4°C for 1 h. The cells were washed again and blocked with PBS containing 0.5% BSA and 0.05% Tween 20 at +4°C for 1 h, then washed once more and incubated with primary antibodies in a 1/10 dilution in PBS. Primary antibodies used were rabbit anti-Tat, anti-Rev, and anti-Nef as well as mouse mabs against Tat, Rev, and Nef (Ovod *et al.*, 1992; Ranki *et al.*, 1994). The cells were incubated at 37°C for 1 h before being washed with PBS and blocked for 5 min at +4°C with PBS containing 0.5% BSA and 0.05% Tween 20. The cells were incubated with either FITC-conjugated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulins or FITC-conjugated $F(ab')_2$ fragment of swine anti-rabbit immunoglobulins (DAKO A/S) at 37°C for 30 min and then washed as before. The cells were then incubated for 3 min with Evans Blue 0.003%, pH 7.0 and the positive cells were evaluated by UV-light microscopy (Fig. 3).

Quantitative Tat expression

An enzyme-based ELISA was used to assay the amount of CAT enzyme expressed from a CAT-encoding plasmid. The plasmid, pNL*CATw*, carries the 5′and 3′LTR from HIV-1 as promoter and polyA signal and thus needs the Tat protein for expression of the encoded gene. HeLa cells (200,000) were plated with complete Eagle's MEM 1× (2 mM L-glutamine, 0.8 g/L sodium bicarbonate, 50 μg/L penicillin/streptomycin, and 10% FCS) (Life Technologies Inc.) and incubated at 37°C in 5% CO_2 until co-transfection 24 h later. Co-transfection was performed with FuGENE 6 transfection reagent (Roche Diagnostics Corp.) as recommended by the manufacturer. FuGENE 6 transfection reagent (5 μl) was used for each sample. The plasmid DNA encoding the *tat* genes was mixed (amounts used were 1, 0.1, 0.01, and 0.0001 μg) with the pNL*CATw* (1 μg) plasmid. The DNA amount was adjusted to a total of 2 μg for each transfection by addition of the noncoding plasmid pBluescript. The pNL*CATw* (1 μg) was also used as a control for unspecific CAT expression. The transfected cells were incubated at 37°C in 5% CO_2 for 24 h before harvesting of the cells. The transfected cells were washed twice with ice-cold PBS before addition of 500 μl of harvesting buffer (0.5% Triton X-100, 100 mM Tris–HCl, pH 7.4). The cells were scraped and transferred to separate tubes in which they were freeze-thawed by incubating them on dry ice for 5 min, followed by 5 min at 37°C and vortexing for 10 s. This was performed three times before the cell debris was separated from the CAT-containing supernatant by centrifugation at 4°C for 15 min at full speed. The samples were diluted 1:100 and 1:1000 in 100 mM Tris–HCl, pH 7.5. The amount of CAT expressed was immediately assayed by a CAT ELISA (Roche Diagnostics Corporation, Roche Molecular Biochemicals) as described by the manufacturer. Briefly, a standard (1, 0.5, 0.25, 0.125, and 0 ng), consisting of recombinant CAT enzyme from *E. coli*, was prepared with 100 mM Tris–HCl, pH 7.5. The standard is contained in the kit. Two hundred microliters of the standard, cell supernatants, and diluted samples was added in duplicates to the anti-CAT-antibody-coated microtiter plate. The plate was incubated at 37°C for 1 h. The plates were washed five times before 400 ng anti-CAT-DIG antibodies were added to each well and the plate was re-incubated for 1 h at 37°C. Again, the plate was washed as above, and 30 mU anti-DIG-POD antibodies were added. The plate was incubated again as above and washed again before POD substrate was added. The plate was then incubated for 20 min at RT before the absorbence was measured at 405 nm (reference, 490 nm). A standard curve was drawn using linear regression analysis. The concentration of the sample was calculated according to: $y = A + B^*x$, where y is the absorbence value, A the value where the standard curve crosses the y axis, B the slope of the curve, and x the concentration of the sample.

Quantitative Rev expression

An antibody-mediated ELISA was used to assay the amount of Gag (p24) protein expressed from a *gag*-encoding plasmid. The *gag*-encoding plasmid, pNL*gagSty330*, carries the 5′ and 3′ HIV LTR as promoter and polyA signal, respectively, resulting in Rev and Tat dependence for expression of the encoded *gag*. HeLa cells (200,000) were plated with complete Eagle's MEM 1× [2 mM L-glutamine, 0.8 g/L sodium bicarbonate, 50 μg/L penicillin/streptomycin, and 10% fetal calf serum (FCS)] (Life Technologies Inc.) and incubated at 37°C in 5% CO_2 until transfection 24 h later. Co-transfection was performed with FuGENE 6 transfection reagent (Roche Diagnostics Corporation, Roche Molecular Biochemicals) as recommended by the manufacturer. FuGENE 6 transfection reagent (5 μl) was used for each sample. The plasmid DNA encoding the *rev* genes was mixed (amounts used were 1, 0.1, 0.01, 0.001, and 0.0001 μg) together with pNL*gagSty330* (0.5 μg) and HCMV*tat* (0.5 μg) encoding the Tat protein. The pNL*gagSty330* was also used alone as a negative control (0.5 μg) for unspecific *gag* production. The plasmid pB*rev* (the *rev* gene under the control of HIV-1 LTR) was included as a positive control. The DNA amount was adjusted to a total of 2 μg for each co-transfection by addition of the noncoding plasmid pBluescript. The transfected cells were incubated at 37°C in 5% CO_2 for 24 h before harvesting of the cells. The transfected cells were washed twice with ice-cold PBS before addition of 500 μl of harvesting buffer (0.5% Triton X-100, 100 mM Tris–HCl, pH 7.4). The cells were scraped and transferred to separate tubes in which the cells were freeze-thawed by incubating them on dry ice for 5 min, followed by 5 min at 37°C and vortexing for 10 s. This was performed three times before the cell debris were separated from the *gag* (p24) containing supernatant by centrifugation at 4°C for 15 min at full speed. The amount of Gag expressed was immediately assayed by an HIV-1 p24 ELISA. Microtiter plates (Nunc-Immuno plate, MaxiSorp, Nalge Nunc International, Denmark) were coated with 20 μg/ml of polyclonal rabbit IgG anti-HIV-1 core antigen (p24) in 0.1 M $NaHCO_3$ buffer, pH 9.6, and incubated at RT overnight and stored at +4°C until use. The plates were washed four times with 0.15 M NaCl with 0.05% Tween 20 (washing buffer). A standard curve was prepared through dilution (in PBS including 0.02% merthiolate, 0.5% BSA and 0.05% Tween 20 and 0.5% Triton X-100) of an HIV-1 virion lysate. Concentrations used were from 2 down to 0.06 ng/ml; 0.1 ml of all samples, the standard curve, cell supernatants, a negative control, and a positive control, was added in duplicates to the wells in the plate. The plate was covered with a sealing tape and incubated at RT over night.

The plate was washed four times with washing buffer, and 0.1 ml of three pooled HRP-conjugated mab anti HIV-1 p24 antibodies was added to each well. The conjugate was diluted in PBS with 0.02% merthiolate, 0.5% BSA, and 0.05% Tween 20 with 2% goat serum (optional). The plate was incubated for 2 h at 37°C and washed as above. A substrate solution ((0.1 ml) of 1.2 mg/ml *o*-phenylenediamine (OPD) in 0.05 M citrate buffer, pH 5.6, with 0.01% H_2O_2, was added to each plate. After 30 min, the reaction was terminated with 0.1 ml 2.5 M H_2SO_4, and the absorbence was measured at 490 nm (reference wavelength, 650 nm). A standard curve was drawn using linear regression analysis and the concentration of the sample was calculated according to: $y = A + B*x$, where y is the absorbence value, A the value where the standard curve crosses the y axis, B the slope of the curve, and x the concentration of the sample.

Quantitative Nef ELISA

HeLa cells (200,000) were plated with complete Eagle's MEM 1× [2 mM L-glutamine, 0.8 g/L sodium bicarbonate, 50 μg/L penicillin/streptomycin, and 10% fetal calf serum (FCS), Life Technologies Inc.] and incubated at 37°C in 5% CO_2 for 24 h before transfection. Transfection was performed with FuGENE 6 transfection reagent (Roche Diagnostics Corp.) as recommended by the manufacturer. FuGENE 6 transfection reagent (5 μl) was used for each sample. The plasmid DNA encoding the *nef* genes was mixed (amounts used were 2, 1.5, 1.0, 0.5, and 0.25 μg) with pBluescript to adjust the total DNA amount to a total of 2 μg for each transfection. Empty vector (pKCMV) and untransfected cells were used as negative controls, HCMV*nef* as a positive control. The transfected cells were incubated at 37°C in 5% CO_2 for 24 h and then washed twice with ice-cold PBS before the addition of 500 μl of 5XRIPA buffer (50 mM Tris buffer pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). The cells were scraped and transferred to separate tubes in which they were freeze-thawed by incubating them on dry ice for 5 min, followed by 5 min at 37°C and vortexing for 10 s. This was performed three times before separating the cell debris from the Nef-containing supernatant by centrifugation at 4°C for 15 min at full speed. A microtiter plate (Nunc-Immuno plate, MaxiSorp, Nalge Nunc Internationa) was coated with 1 μg/ml goat anti-mouse IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL) in 50 mM potassium phosphate buffer, pH 7.2 (PB buffer) overnight at RT. The plate was washed three times in PB buffer with 200 mM NaCl (PB-NaCl buffer) and saturated for 1 h at RT with PB–NaCl buffer with 1% BSA. It was then washed three times in PB–NaCl buffer with 0.1% Triton X-100 before overnight incubation at +6°C with PB–NaCl buffer with 1% sucrose and 1% BSA. The plate was emptied and dried overnight at +6°C. Mouse anti-Nef Mab (Ovod *et al.*, 1992; Ranki *et al.*, 1994) was diluted 1/100 in PB–NaCl buffer with 1% BSA and incubated at +6°C overnight. The plate was washed three times with 0.5 M KCl, followed by three times with deionized water. It was then blocked at +6°C overnight with PB–NaCl buffer with 1% sucrose and 1% BSA. The plate was emptied and dried at RT for 1 h. The Nef-containing samples, the cell supernatants, and a standard curve consisting of recombinant Nef (kindly provided by B. Kohlesein and V. Erfle, GSF, Germany) in PB–NaCl with 1% BSA ranging from 50 to 0.78 ng/ml were added to the plate and incubated at 37°C for 2 h. The plate was washed three times with 0.5% Triton X-100 in deionized water. Polyclonal rabbit anti-Nef IgG was diluted 1/100 in PB–NaCl buffer with 1% BSA and incubated on the plate overnight at RT. The plate was washed three times with 0.5% Triton X-100 in deionized water, followed by the addition of horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (DAKO A/S), diluted 1/2000 in PB–NaCl buffer with 1% BSA, at 37°C for 1.5 h. The plate was washed as before and 0.1 ml of a substrate solution of 1.2 mg/ml *o*-phenylenediamine (OPD) in 0.05 M citrate buffer, pH 5.6, with 0.01% H_2O_2, was added. After 10 min, the reaction was terminated with 0.1 ml 2.5 M H_2SO_4, and the absorbence was measured at 490 nm (reference wavelength, 650 nm). A standard curve was drawn using linear regression analysis. The concentration of the samples was calculated according to: $y = A + B*x$, where y is the absorbence value, A the value where the standard curve crosses the y axis, B the slope of the curve, and x the concentration of the sample.

Immunizations

Ten to 12-week-old (C57BL/6) H-2b mice transgenic for human A2.01 were immunized twice at Weeks 0 and 4 with the corresponding DNA administered intramuscularly by injection in the quadriceps muscle in both hind legs. All plasmids were diluted in water. For single gene administration (Tat $n = 8$, Rev $n = 8$, and Nef $n = 7$), the total DNA amount used was 10–50 μg at each immunization. These doses have been shown to be effective in raising a Tat-, Rev-, and Nef-specific immune response (Hinkula *et al.*, 1997b). For combined gene immunization, the mice ($n = 18$) were immunized twice at Weeks 0 and 4 with 3 × (10–50) μg DNA at each immunization. The DNA mixture was prepared from equal amounts of each of the three plasmids to keep the gene content constant in respect to the single-gene immunizations. Each mouse in the combined gene immunization group received 20–100 μg of each gene. The empty vector, pKCMV, was included as a control, using a 2 × 150 μg dose. Mice were killed at Week 6. Blood was taken prior to the first immunization and 2 weeks after each immunization.

Antigens

The antigens used were recombinant Tat protein (Cecilia Svanholm, MTC, Karolinska Institute, Sweden), recombinant Rev (in-house production) expressed in a baculovirus system, and affinity-purified recombinant Nef antigen produced in *E. coli* (kindly provided by B Kohlsein and V Erfle, GSF, Germany). The synthetic peptides used were 20-mers with a 5-amino acid (5aa) overlap covering the HIV-1 LAI strain regulatory proteins Tat, Rev, and Nef (Accession No. K02013) (Myers *et al.*, 1994). The peptides were synthesized according to the 9-fluorenylmethoxycarbonyl method. Recombinant Tat and Nef (0.5μg/ml) and 1 μg/ml of Rev were used for coating ELISA plates and for T-cell stimulation. Synthetic peptides were used at a concentration of 5 μg/ml, and concanavalin A (Con A) was also used at a 5μg/ml concentration Aldrich Inc., Saint Louis, MO).

B-cell stimulation *in vitro* assay

Spleen cells (100,000/well) in 200 μl of RPMI 1640 supplemented with 5% inactivated fetal calf serum (FCS) were cultured for 72 h at 37°C in 96-well flat-bottomed cell culture plates (Nunc) with stimulating peptide antigens (10 μg/ml) or recombinant protein (1 μg/ml). The plates were pre-coated with recombinant Tat, Rev, and Nef or Tat, Rev, and Nef peptides, diluted in 0.05 M sodium carbonate buffer (pH, 9.5–9.6), and incubated at RT overnight. After incubation, the plates were washed and goat anti-mouse IgG (H+L) HRP labeled (Bio-Rad Laboratories) were diluted 1/3000 in PBS with 0.5% BSA and added to the plates. The plates were incubated for 60 min at 37°C before being washed with 0.9% sodium chloride with 0.05% Tween 20. Hydrogen peroxidase activated orto-phenylenediamine (OPD; Aldrich) in 0.1 M citric acid (pH 5.5) was added as a substrate. Color development was terminated with 100 μl of 2.5 M sulfuric acid per well after 20-min incubation at RT. The absorbence was measured at 490 nm (reference wavelength, 650 nm). Specific, positive stimulation was assessed as optical density (OD) values higher than the cut-off value (mean value of the control group plus 2 SD).

Measurement of antibody responses

For detection of specific antibody responses, ELISA specific for Tat, Rev, and Nef was used. Recombinant proteins or representative peptides were diluted in 0.05 M sodium carbonate buffer (pH, 9.5–9.6) and used for coating 96-well plates (Nunc-Immuno plate, MaxiSorp, Nalge Nunc International). Peptides were coated at a concentration of 10 μg/ml and recombinant proteins at 1 μg/ml for Tat and 0.5 μg/ml for Rev and Nef. Peptides used for peptide ELISA were as follows: Tat peptides representing aa 31–50 and aa 46–65; Rev peptides representing aa 1–20, aa 16–35, and 31–50; Nef peptides representing 151–[illegible]aa 166–185, and 181–205. The plates were sealed and incubated overnight at RT before being washed four times with washing buffer (0.9% NaCl with 0.05% Tween 20) and blocked with 1% BSA in PBS for 1 h at 37°C. Mouse sera were diluted 1/100, 1/300, 1/900, and 1/2700 in PBS with 0.5% BSA and 0.05% Tween 20. Serum dilution (100 μl) was added per well, and the plates were incubated at 4°C overnight, followed by washing as above. HRP-labeled goat anti-mouse immunoglobulin G (DAKO A/S) was diluted 1/2000. The plates were incubated for 60 min at 37°C before being washed as above. Hydrogen peroxidase activated OPD in 0.1 M citric acid (pH 5.5) was added as a substrate. Color development was terminated with 100 μl of 2.5 M sulfuric acid per well after 30-min incubation at RT. The absorbence was measured at 490 nm (reference wavelength, 650 nm). Samples were considered positive when the optical density (OD) value was higher than the cut-off value (geometric mean value of the naive control group plus 2 SD). Measurement of B-cell stimulation *in vitro* is considered to be a more specific and sensitive measurement of humoral responses than measurement of IgG secretion.

T-cell proliferation assay

Spleen cells (200,000 per well) were cultured, in triplicates per sample, for 5–6 days in RPMI 1640 supplemented with 4 mM L-glutamine, 50 IU of penicillin per ml, and 50 μg of streptomycin per ml. Also included was 10% fetal calf serum (Life Technologies Inc.) in the presence of 0.1–1 μg of antigen, mitogen, control antigen, and medium. A 50-μl volume of ^{3}H-labeled thymidine was added per well (1 μCi), the cells were incubated for 16 h, and thymidine incorporation was measured in a β-counter. The mean radioactivity (counts per minute, cpm) was calculated for all triplicates of antigens, control antigen, mitogen, and medium control. To obtain a value for specific proliferation (stimulation index, SI), the mean cpm value for each antigen was divided by the cpm value for medium. Stimulation was considered positive if the SI value was 3. All mice with a SI value for ConA <5 were excluded.

Subclass determination

For IgG subclass determination, a subclass specific ELISA was performed according to the manufacturer (Sigma, St. Louis, MO). Plates and sera were prepared and incubated as above. After incubation of sera overnight at 4°C, the plates were washed as above and incubated for 2 h at 37°C with subclass specific antibodies diluted 1:1000 in PBS with 0.5% BSA and 0.05% Tween 20. The plates were washed as above. HRP-labeled rabbit anti-goat IgG (DAKO A/S) was diluted 1/1500 and added to the plates, which were incubated for 60 min at 37°C before being washed as above. Hydrogen-peroxi-

dase-activated OPD in 0.1 M citric a[redacted](pH 5.5) was added as a substrate. Color develop[redacted]nt was terminated with 100 μl of 2.5 M sulfuric acid per well after 30-min incubation at RT. The absorbence was measured at 490 nm (reference wavelength, 650 nm) and the IgG1 to IgG2a ratios were determined.

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